REMARKS/ARGUMENTS

Reconsideration of this application is requested. Claims 1-17 are in the case.

I. THE SPECIFICATION

The specification has been further amended to include a sequence listing identifier at page 32, line 25. No new matter is entered.

II. THE RESTRICTION REQUIREMENT

In response to the restriction requirement, Applicants hereby elect Group IV, claim 10, drawn to a method for inhibiting or reducing the proliferation of prostate cancer cells, or to a method for treating the proliferation of prostate cancer cells, or to a method for treating prostate cancer, said method comprising administering to the cells or to a subject in need of treatment a **s**PLA_2-IIA inhibitor, wherein said **s**PLA_2-IIA inhibitor is c(2NapA)LS(2NapA)R. The election is made with traverse.

Groups II to IV (claim 10) are categorized as being directed to administration of **c**PLA_2-IIA inhibitors (i.e., cytosolic PLA_2-IIA inhibitors). In fact, these claims are directed to inhibitors of the **secreted** form of PLA_2 not the cytosolic form, so they are **s**PLA_2-IIA inhibitors. This is a critical point because the citation raised in justifying the Restriction Requirement (Hermann et al., 1997, a copy of which is enclosed) discloses so-called "selective" inhibitors of **c**PLA_2.

Further, subsequent published work on the inhibitor disclosed in Hermann et al. (AACOCF3) has shown that it is not even specific for $cPLA_2$ (see Meyer et al. 2005, a copy of which is enclosed). In fact, the inhibitor inhibits several other enzymes.

In light of the above, it is clear that at least all of groups II to IV should be considered in the current application, and that claims 5-9 are clearly linking claims. There is nothing in the cited prior art to discloses a method of reducing the proliferation of prostate cancer cells by administering an **s**PLA_2-IIA inhibitor. Modification of the Requirement at least to the extent noted above is respectfully requested.

Favorable action on this application is awaited.

Respectfully submitted,

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Attachments: Meyer, et al; *Current Pharmaceutical Design*, 11, 1301-1312 (2005)
Herrmann, et al; *Experimental Cell Research*, 234, 442-451 (1997)

EXPERIMENTAL CELL RESEARCH 234, 442–451 (1997)
ARTICLE NO. EX973653

Regulation of Lipid Signaling Pathways for Cell Survival and Apoptosis by bcl-2 in Prostate Carcinoma Cells

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Compelling evidence indicates that activation of the JNK/SAPK signaling pathway is obligatory for apoptosis induction by multiple cell stresses that activate the sphingomyelin cycle. Moreover, ectopic expression of bcl-2 can impair apoptosis signaling by most of the cell stresses that activate the ceramide/JNK pathway. Here we show that enforced expression of bcl-2 protects prostate carcinoma cells against the induction of apoptosis by exogenous C_2-ceramide. Moreover, enforced bcl-2 expression blocked the capacity of C_2-ceramide to activate JNK1, indicating bcl-2 functions at the level of JNK1 or upstream of JNK1 in the ceramide/JNK pathway. The contribution of bcl-2 to the regulation of the arachidonate pathway for prostate carcinoma cell survival was also investigated using highly selective inhibitors of arachidonate metabolism. Our results indicate bcl-2 can protect cells against diminished availability of arachidonic acid, 12-HETE, and 15-HETE. Finally, arachidonic acid substantially suppresses the induction of apoptosis by C_2-ceramide, providing evidence for the opposing influences of these lipid signaling pathways in the mediation of prostate carcinoma cell survival. These results provide evidence for opposing influences of the ceramide and arachidonate signaling pathways in the mediation of cell death and cell survival, respectively, in prostate carcinoma cells and suggest a dual role for bcl-2 in this context. © 1997 Academic Press

INTRODUCTION

The bcl-2 oncogene was identified by virtue of its association with the $t(14;18)$ translocation in follicular lymphomas [1–3]. The contribution of bcl-2 to oncogenesis has been shown to result from its capacity to extend cell survival by blocking or delaying the onset of apoptotic cell death [4–7]. It is now recognized that bcl-2 is expressed in a variety of human tumors [8–10]. In particular, bcl-2 overexpression is associated with the progression of epithelial malignancies including carcinoma of the prostate [11–13].

It has been shown that bcl-2 can impair cellular pathways for death that are seemingly independent including, but not limited to, TNFα-mediated cytotoxicity, interleukin 1β-converting enzyme (ICE), peroxides and reactive oxygen species, growth factor withdrawal, γ-radiation and UV-radiation, p53, c-myc, calcium, adriamycin, androgen withdrawal, neurotrophin withdrawal, and anti-CD3 receptor clustering [8, 14]. The fact that bcl-2 can protect against multiple cell death signals suggests the possibility that bcl-2 may be regulating a common cell death pathway and function at a point of signaling convergence [15]. In this report, we investigate bcl-2 in the context of two bioactive lipid signaling pathways commonly activated (ceramide) or repressed (arachidonic acid) by these cell stresses.

The many cellular activities of arachidonic acid (AA), a polyunsaturated fatty acid, are initiated following its release from plasma membrane phospholipids following engagement of G-protein-coupled membrane receptors by ligand [16]. Activation of $cPLA_2$ and AA release has been reported following treatment with epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), interferon-α and interferon-γ, serotonin, and TNFα [17–23]. Once generated, AA may be shunted into four principal pathways; (1) it can be reincorporated into membrane phospholipid pools, (2) it can metabolized via the lipoxygenase (LOX) pathway leading to the biosynthesis of leukotrienes (LTs), hydroxyeicosatetraenotes (HETEs), and epoxyhydroxides, (3) it can be enzymatically converted by cytochrome P-450 to epoxyeicosatrienoic acids (EETs), diols, and HETEs, and (4) it can be converted in a series of reactions initiated by cyclooxygenase (COX) to prostaglandins (PGs), thromboxanes (TXs), and prostacyclins. Once synthesized, these eicosanoids participate in mediating nu-

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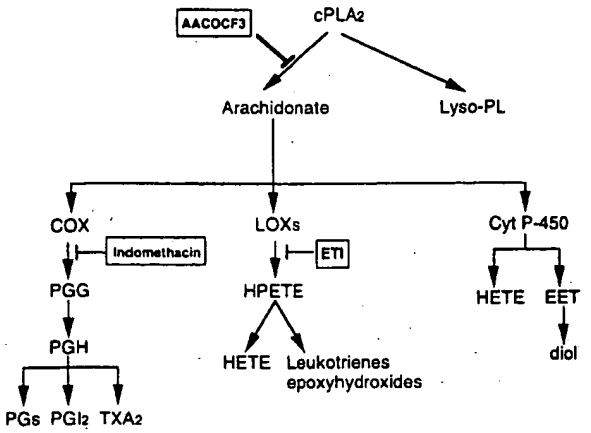

FIG. 1. Schematic depiction of the arachidonate/eicosanoid metabolism pathway. The site of action of the AACOCF3, ETI, and Indomethacin, inhibitors of PLA_2, LOX, and COX, respectively, is indicated.

merous cellular properties, including cell motility, adhesion, proliferation, ion channel behavior, inhibition of Na^+/K^+-ATPase activity, and c-myc gene expression [16]. These pathways are outlined schematically in Fig. 1.

The involvement of lipid second messengers in cell death signaling is supported by studies of receptors and cell stresses that activate the sphingomyelin cycle and generate ceramide. Ceramide and its targets have been implicated in the mediation of a program for apoptosis in response to a variety of external cell stresses such as TNFα, UV- and γ-radiation, H_2O_2, and heat shock [24, 25]. Importantly, it has been reported that cell death signaling by ceramide is apparently executed by prompt activation of the MEKK/JNKK/JNK pathway, in that, dominant negative mutants of c-jun and SEK-1 (JNKK) diminish the extent of ceramide-induced apoptosis in bovine aortic endothelial cells and human U937 monoblastic leukemia cells [25].

By using selective inhibitors of AA metabolism we determined that HETEs derived via the LOX pathway are required for the survival of human and rat prostatic carcinoma cells. Conversely, PGs do not appear to signal for cell survival in prostatic carcinoma cells. It was also determined that bcl-2 protects prostatic carcinoma cells against impaired HETE biosynthesis. The protective effect of bcl-2 against C_2-ceramide-induced apoptosis was confirmed in prostate carcinoma cells and shown to be associated with a block in JNK1 activation. Finally, evidence is provided that exogenously added AA can significantly block the induction of apoptosis by C_2-ceramide. This result argues that the AA and ceramide pathways represent opposing bioactive lipid signaling systems for prostate carcinoma cell survival that are governed by bcl-2.

Our results using selective inhibitors of arachidonate metabolism offer significant insight into bcl-2 function and suggest a mechanism by which bcl-2 may protect cells from cell death induction under conditions of limiting growth factor availability or growth factor deprivation. Our results also define the stress-activated protein kinase JNK1 as one regulatory target for bcl-2 in the ceramide pathway for apoptosis. By imposing a block to JNK1 activation, bcl-2 might be strategically positioned to regulate multiple cell stresses that activate the sphingomyelin cycle for ceramide synthesis.

METHODS

Reagents. Calcein AM and ethidium homodimer-1 were obtained from Molecular Probes (Eugene, OR). RPMI medium and fetal bovine serum were purchased from Gibco/BRL (Gaithersburg, MD). The Δ21 anti-bcl-2 polyclonal affinity-purified antibody, the anti-JNK1-agarose conjugate, and the JNK [G-7] antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Purified GST-c-jun (169) protein was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Zymed (South San Francisco, CA). Nitrocellulose membranes were purchased from Micron Separations, Inc. (Westborough, MA). The Enhanced Chemiluminescence (ECL) detection system was obtained from Amersham (Arlington Heights, IL). Arachidonic acid was purchased from Sigma (St. Louis, MO). C_2-ceramide, ETI, and indomethacin were obtained from Biomol (Plymouth Meeting, PA). Calphostin C and TPA were purchased from Calbiochem (La Jolla, CA).

Cell lines and culture conditions. The prostatic carcinoma cell lines, LNCaP and LNCaP-bcl-2, Dunning-G-C1 and Dunning-G-bcl-2, were cultured in RPMI medium supplemented with 10% fetal bovine serum (complete medium). LNCaP-bcl-2 and Dunning-G-bcl-2 were derived from their respective parental cell lines by stable transfection with a Splenic Focus Forming Virus-based expression vector containing a complete human bcl-2 cDNA or empty vector as a negative control. Stable transfectants were selected in growth medium supplemented with 400 μg/ml G418. Colonies were selected using cloning cylinders. All selected bcl-2 expressing subclones were found to be stable and constitutively express high levels of bcl-2 after removal of G418, even through multiple passages. For this reason, all experiments were performed on cells cultured in culture medium without G418 and the bcl-2 expression profile was monitored (by immunoblotting) in parallel with any experiments assesing bcl-2 survival signaling. Cells were maintained in a 5% humidified CO_2 chamber and subcultured (1:10 split) once per week by typsinization (0.25% trypsin in calcium and magnesium-free phosphate-buffered saline). For all experiments, the cell lines were used at low passage numbers.

Cell viability assays. Cells were seeded at 1.0×10^5 cells per well in 48-well plates. After 3–5 days of culture in complete medium these cells were washed once with RPMI containing 0.5% FBS. Cells were then treated with the appropriate inhibitor (ETI, $AACOCF_3$) or C_2-ceramide prepared fresh in ethanol and diluted into RPMI containing 0.5% FBS. All experiments with ETI, AA, and $AACOCF_3$ were performed in minimal lighting conditions to minimize photoinactivation. Each treatment group was performed in triplicate and in RPMI containing 0.5% FBS. After 24 h the cells were washed with complete medium and then incubated with complete medium containing 1 μg/ml CAM with or without ETHD. Cells were loaded with CAM for 20 min at 37°C, washed twice, and refed with complete medium. For assays using ETHD, the nuclear staining pattern was monitored using a 580-nm emission filter by standard epifluores-

cence. Measurement of intracellular calcein was performed using a Cytofluor model 2350 (Millipore) fluorometric microplate reader using a 485-nm excitation filter and a 530 emission filter set.

Immunoblot analysis. Subconfluent cells, cultured without G418, were washed once with PBS and lysed directly on the plate with 2× SDS–PAGE sample loading buffer. Cell lysis volumes were adjusted to reflect cell counts taken from other plates. DNA was sheared by 10 passages through an 18- and a 25-g needle. Samples were boiled for 5 min and centrifuged at 10,000*g* for 5 min to remove any particulate material. Proteins were resolved on 12.5% polyacrylamide gels and electroblotted to a nitrocellulose membrane. Membranes were blocked in PBS containing 0.1% Tween 20 and 4% nonfat dry milk. Blocked membranes were probed with an anti-bcl-2 monospecific polyclonal primary antibody (1:5000), reactive with the human and rat bcl-2 protein, for 1 h at room temperature, washed four times for 15 min per wash and probed with secondary goat anti-rabbit IgG coupled to horseradish peroxidase. Detection of bound secondary antibody was performed using Enhanced Chemiluminescence (ECL) as per the manufacturer's directions.

Determination of c-jun N-terminal kinase 1 (JNK1) activity. LNCaP-C or LNCaP-bcl-2 cells were treated with C_2-ceramide (50 or 100 μM) for 40 and 80 min in RPMI supplemented with 0.5% FBS. At the indicated times, the medium was removed, the cells were washed once with PBS and the cell monolayer was lysed in JNK immunoprecipitation buffer (20 m*M* Hepes, pH 7.4, 2 m*M* EGTA, 1 m*M* DTT, 1 m*M* Na_3 VO_4, 1% Triton X-100, 10% glycerol, 2 μM leupeptin, 10 μg/ml soy bean trypsin inhibitor, and 400 μM PMSF). The volume of JNK immunoprecipitation buffer used was adjusted to reflect equivalent cell numbers. After 10 min on ice, the insoluble material was removed by centrifugation at 10,000*g* for 5 min. The clarified supernatant was decanted to a fresh tube and 20 μl of a slurry of anti-JNK1 antibody-conjugated Sepharose beads (Santa Cruz Biotechnologies) was added. The mixtures were mixed with gentle agitation for 4 h at 4°C. The immunecomplex was collected by centrifugation (1,500*g* for 5 min) and the beads were washed four times in 1 ml of JNK immunoprecipitation buffer. The final bead pellet was resuspended in 50 μl assay buffer (20 m*M* $MgCl_2$, 20 m*M* Hepes, pH 7.4). To 25 μl of the immunecomplex in assay buffer was added 0.8 μg (4 μl) purified c-jun(1-169)-GST and 1 μl [γ-^{32}P]ATP (3,000 Ci/mmol). The kinase reaction was performed at 30°C for 20 min and terminated by the addition of 1 vol of 2× Laemli buffer. The phosphorylated products were separated on a 12.5% gel and detected by autoradiography.

As an alternative means of determining JNK1 activation, a phospho-specific antibody that recognizes catalytically activated JNK1 phosphorylated at Thr-183 and Tyr-185 (JNK [G-7]) on immunoblots was used. This antibody was raised against an epitope mapping to amino acids 183–191 phosphorylated on Thr-183 and Tyr-185 and is non-cross-reactive with ERK1, ERK2, or p38 MAPK. Cell extracts were prepared from C_2-ceramide-stimulated cells (20 μM) or controls (ethanol) in 2× SDS–PAGE sample loading buffer containing 1 m*M* sodium orthovanadate. The cell extracts were immunoblotted as described above and probed with the mouse monoclonal anti-JNK [G-7] antibody. Bound JNK [G-7] antibody was detected as described above using an affinity purified horseradish peroxidase-conjugated goat anti-mouse antibody.

RESULTS

Generation of bcl-2 expressing clones of LNCaP and Dunning-G prostatic carcinoma cell lines. LNCaP and Dunning G prostatic carcinoma cells were transfected with an expression vector containing a full-length human bcl-2 cDNA. For each cell line, vector control clones (LNCaP-C and Dunning G-C1) transfected with

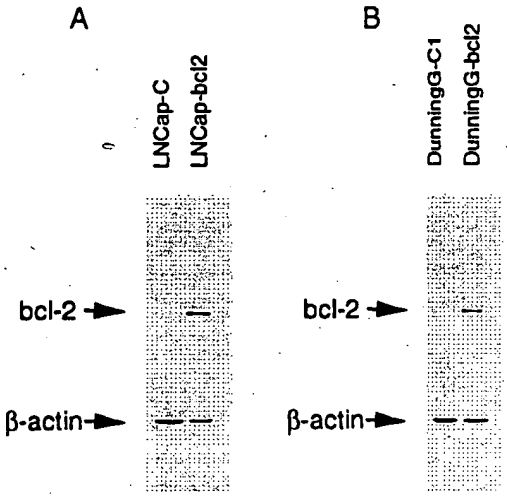

FIG. 2. Bcl-2 overexpression in LNCaP-bcl-2 prostatic carcinoma cells (A) and Dunning-G-bcl-2 cells (B). Human LNCaP and rat Dunning G prostatic carcinoma cells were stably transfected with a Splenic Focus Forming Virus plasmid expression vector containing a full-length human bcl-2 cDNA or empty vector DNA. Stable transfectants were analyzed for bcl-2 protein by immunoblot analysis using an affinity-purified anti-bcl-2 polyclonal antibody (Δ21). Primary antibody was detected using a goat anti-rabbit polyclonal antibody conjugated to horse radish peroxidase followed by ECL. Gel loading was based upon cell equivalents and loading monitored by immunoblot analysis of cell extracts using a β-actin antibody.

empty vector DNA were compared to a corresponding bcl-2 transfectant (LNCaP-bcl-2 and Dunning G-bcl-2) by immunoblot analysis (Fig. 2). These and similar bcl-2 congenic cell lines have previously been used to demonstrate the contribution of bcl-2 to androgen-independent growth [26] and resistance to chemotherapeutic cell death induction [27].

Bcl-2 expression in prostatic carcinoma cells protects against AACOCF$_3$-mediated apoptosis. Cells were grown in RPMI supplemented with 10% FBS then switched to RPMI containing 0.5% FBS at the onset of the experiment. Cells were then treated with 5 to 20 μM AACOCF$_3$, a potent and selective inhibitor of cPLA$_2$ [28–30; Fig. 1] or ethanol solvent alone (controls) in RPMI containing 0.5% FBS. After 24 h, the assay was terminated by washing the wells and replacing with RPMI supplemented with 10% FBS and CAM (1 μg/ml). Cells were loaded with CAM, washed, and analyzed for calcein-generated fluorescence, as an indicator of cell viability. CAM is an acetoxymethyl ester derivative of calcein that is readily plasma membrane permeable and cleaved by nonspecific estarases to release calcein, a pH-insensitive indicator of cell viability that is retained well by viable cells. Cells that have lost plasma membrane integrity fail to retain calcein and are detected by virtue of their diminished fluorescent inten-

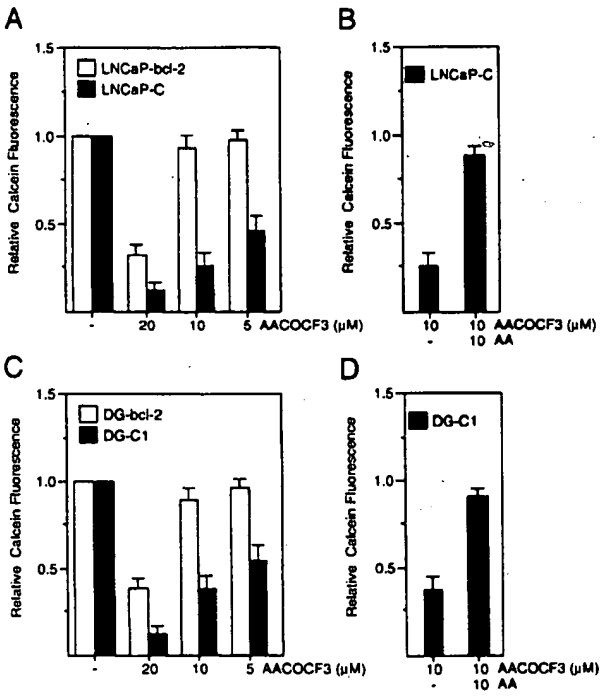

FIG. 3. Dose-dependent inhibition of prostatic carcinoma cell viability by $cPLA_2$-selective inhibitor $AACOCF_3$: Protection conferred by bcl-2. Protection from cell death induction by $AACOCF_3$ was conferred by elevated bcl-2 expression (A and C) and exogenous AA (B and D). Cells were treated with ethanol or various doses of $AACOCF_3$ prepared in ethanol. All treatments with $AACOCF_3$ and AA were performed in triplicate in the presence of 0.5% FBS. After 24 h the cells were loaded with CAM (1 μg/ml) and assessed for conversion of CAM to calcein using a Cytofluor fluorometric microplate reader. $AACOCF_3$ treatment was also performed on LNCaP-C and Dunning-G-C1 control cell lines pretreated with AA (B and D). Data are presented as relative fluorescence intensity of calcein and normalized to control cells. Columns, mean; bars, standard deviation.

sity by fluorescence microscopy or spectrofluorometry. The use of CAM (and ETHD described below) to assess cell death by apoptosis has been described by other researchers (31, 32). Enforced expression of bcl-2 protein in both the LNCaP (Fig. 3A) or Dunning-G (Fig. 3C) prostatic carcinoma cell lines provided a significant protective effect against apoptosis induction by 10 μM $AACOCF_3$ ($P \leq 0.005$). To ensure the selectivity of $AACOCF_3$ for $cPLA_2$ inhibition, cells were also treated with $AACOCF_3$ in the presence of exogenous AA. Coincubation with exogenous AA effectively limited cell death induction by $AACOCF_3$ (Figs. 3B and 3D).

A selective lipoxygenase inhibitor, but not a cyclooxygenase inhibitor, induces apoptosis in prostatic carcinoma cells. In order to assess the potential input of downstream effectors of AA metabolism, selective inhibitors of the lipoxygenase and cyclooxygenase pathway were examined. To preferentially impair lipoxygenases (LOXs), ETI (5, 8, 11-eicosatriynoic acid) was used. ETI is a selective inhibitor of 5-, 12-, and 15-LOXs (IC_{50} = 5, 20 and 20 μM for 5-, 12-, and 15-LOX, respectively) versus cyclooxygenase (IC_{50} = 50 μM) in whole cells [33]. To impede the activity of cyclooxygenase (COX) we used indomethacin (Fig. 1), a COX inhibitor [34]. Application of indomethacin in low serum medium up to 40 μM, a concentration sufficient to effectively inhibit COX (IC_{50} = 0.1 μM) and prostaglandin H synthase-1 (IC_{50} = 4.9–8.1 μM) but, not the 5-, 12-, and 15-LOXs (IC_{50} > 100 μM for 5-, 12-, and 15-LOXs) in whole cells [34, 35], did not induce any detectable cell death for either the LNCaP or Dunning-G cells as assessed by CAM uptake, cleavage and retention, or ethidium homodimer (ETHD) uptake (data not shown). ETHD is a fluorescent, cell-impermeant DNA intercalating molecule that is useful when used in conjunction with CAM in the assessment of cell viability since their emission spectra do not overlap. The complete lack of ETHD uptake by viable cells, its large fluorescence shift upon DNA binding, and high affinity for DNA make it extremely useful in identifying apoptotic cells by fluorescence microscopy. Cells that have lost membrane integrity and are truly apoptotic will incorporate ETHD into fragmented chromatin. The ETHD staining pattern will accurately highlight DNA fragmentation associated with apoptosis. Even after long term cultures (1–2 weeks), no cytotoxicity was noted using indomethacin (40 μM) on these cells lines.

Recent studies regarding the structural basis of anti-inflammatory drug action with respect to COX-2 inhibition find that indomethacin binds deeply within the active site of COX-2 [36]. This property of indomethacin, and its slow, time-dependent inhibition of both COX-1 and COX-2 [37], make it an ideal reagent for the experiments in question, where our goal was to achieve a generalized block to prostaglandin biosynthesis. The use of indomethacin is sufficient to inhibit the COX-1, COX-2, and the prostaglandin H synthase-1 pathways for prostaglandin biosynthesis [34–36, 38]. Thus, we believe that it is exceedingly improbable that eicosanoids generated via the COX pathway signal for cell survival in Dunning G or LNCaP prostate carcinoma cells under the described conditions.

Conversely, treatment of cells with ETI resulted in the induction of cell death in both control and bcl-2-expressing prostatic carcinoma cell lines the range of 20–40 μM (Fig. 4). This range of ETI is sufficient to inhibit the 5-, 12-, and 15-LOXs but not COX in intact cells. Yet, since cell death was observed only at >20 μM ETI (Fig. 4), these results most likely indicate that inhibition of 5-LOX is not detrimental to cell viability. Rather, the 20–40 μM range suggests that in order to initiate ETI-mediated apoptosis in these cells the 12- and 15-LOX activities must be inhibited.

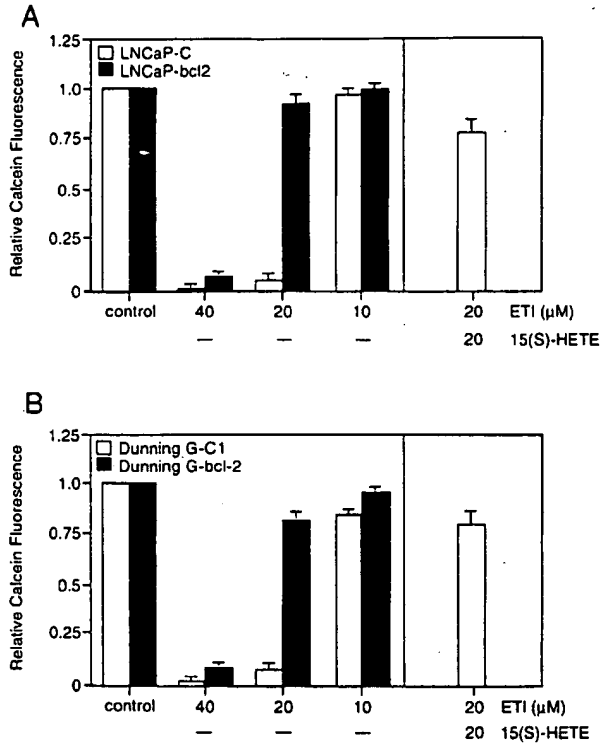

FIG. 4. Induction of apoptosis in prostatic carcinoma cells by a lipoxygenase-selective inhibitor, ETI: Regulation by bcl-2. (A) LNCaP-C (open box) and LNCaP-bcl-2 (closed box) or (B) Dunning-G-C1 (open box) and Dunning-G-bcl-2 (closed box) cells were treated with the indicated concentrations of ETI or ethanol (solvent control) in medium containing 0.5% serum. After 24 h, cell viability was determined by loading the cells with CAM and assessing calcein fluorescence intensity. To assess the specificity of ETI-induced apoptosis, (A) LNCaP-C or (B) Dunning-G-C1 cells were cotreated with ETI (20 μM) and 15(S)-HETE (20 μM) and cell viability monitored by loading the cells with CAM and assessing the relative calcein fluorescence intensity. In this assay, rounded nonadherent cells were removed by washing. These nonadherent cells generally appeared to retain calcein less efficiently than adherent cells by fluorescence microscopy (data not shown). Columns, mean; bars, standard deviation.

Bcl-2 overexpression and exogenous 15-HETE protect against apoptosis induced by ETI. We also examined whether the induction of apoptosis in prostatic carcinoma cells by the lipoxygenase-selective inhibitor ETI could also be affected by expression of bcl-2. Expression of bcl-2 was found to confer an enhanced survival advantage to the LNCaP cells (Fig. 4A, 92.3% viability at 20 μM vs 5.1% viability for control LNCaP cells) and to the Dunning-G cells (Fig. 4B, 82.3% viability at 20 μM vs 8.6% viability for control Dunning-G cells). The selectivity of ETI-mediated apoptosis could also be demonstrated by pretreatment with HPLC purified 15(S)-HETE (20 μM) followed by ETI/15(S)-HETE cotreatment (20 μM each). Such treatment effectively rescued ETI-mediated apoptosis (Fig. 4).

At 40 μM ETI the protective effects conferred by overexpression of bcl-2 is diminished considerably (6% viability at 40 μM vs 92.3% viability at 20 μM for the LNCaP-bcl-2 cells; 7.8% viability at 40 μM vs 82.3% viability for the Dunning-G-bcl-2 cells). At this concentration, however, ETI is not lipoxygenase selective.

AACOCF$_3$-mediated killing of prostatic carcinoma cells occurs through an apoptotic mechanism. To examine the morphological features associated with AACOCF$_3$-induced cell death, the Dunning G-C1 and Dunning-G-bcl-2 cells were preloaded with CAM, washed, and treated with AACOCF$_3$ (50 μM). The appearance of classically apoptotic cells having extensive blebbing of their plasma membrane and cytoplasmic condensation was monitored by epifluorescence microscopy (Figs. 5A–5F) and the percentage of apoptotic cells as a function of time was plotted (Fig. 5G). At a high concentration of AACOCF$_3$, apoptosis was rapidly initiated (100% apoptosis by 60 min for the Dunning-G-C1). In bcl-2 expressing cells the kinetics of apoptosis induction was significantly inhibited relative to control cells ($P \leq 0.01$ at 30 min and $P \leq 0.01$ at 45 min). That the process of cell death induced by AACOCF$_3$ was apoptotic was further supported by staining nuclei with ETHD (data not shown).

Bcl-2 expression in prostatic carcinoma cells protects against C_2-ceramide-mediated apoptosis. Cells were treated with various concentrations of C_2-ceramide or ethanol solvent alone (controls) in RPMI. Cell viability and apoptosis of Dunning-G cells treated with C_2-ceramide was determined by epifluorescence microscopy of CAM- and ETHD-loaded cells (Figs. 6A–6D). Expression of bcl-2 in either the LNCaP (Fig. 6E) or Dunning-G (Fig. 6F) cell lines provided a significant ($P \leq 0.01$ and $P \leq 0.001$, respectively) protective effect against apoptosis induction by C_2-ceramide at 10 μM compared to control cells. Similar results were obtained for C_2-ceramide-treated LNCaP cells (Fig. 6G).

Ceramide- and AACOCF$_3$-mediated apoptosis are additive. LNCaP-C cells were simultaneously treated with C_2-ceramide and AACOCF$_3$ to determine whether the ceramide and cPLA$_2$ pathways functioned independently in the regulation of cell death. Cells were assayed for the appearance of the nuclear alterations of apoptosis by ETHD (Fig. 6H). Treatment of cells with C_2-ceramide and AACOCF$_3$ together exhibited a significant and additive effect compared to either AACOCF$_3$ or C_2-ceramide alone. This effect suggests that simultaneous activation of the ceramide pathway (apoptosis) and inhibition of the AA pathway (survival) is a "double hit" that is remarkably detrimental to cell viability.

Ectopic Bcl-2 overexpression diminishes JNK1 activation by ceramide. An immune complex kinase assay

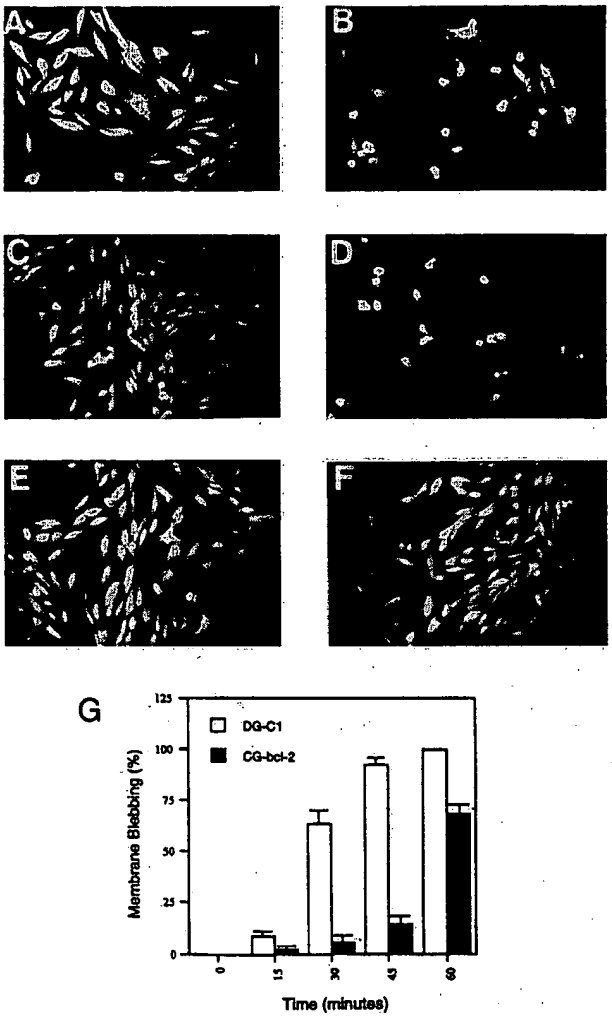

FIG. 5. High dose $AACOCF_3$-induced cell death of Dunning-G prostatic carcinoma cells: Delay of kinetics by bcl-2. Cells were preloaded with CAM and visualized by epifluorescence microscopy after treatment. Dunning-G-bcl-2 cells (A) and Dunning-G-C1 cells (B) treated with 50 μM $AACOCF_3$ for 30 min. Dunning-G-bcl-2 cells (C) and Dunning-G-C1 cells (D) treated with 50 μM $AACOCF_3$ for 40 min. Dunning-G-bcl-2 cells (E) and Dunning-G-C1 cells (F) treated with ethanol alone for 40 min. Apoptosis was quantitated by scoring cells in randomly selected fields (400×). Data represent the percentage of cells displaying morphologic alterations of apoptosis (G).

(ICKA) was used to measure the catalytic activity of immunoprecipitated JNK1 toward purified c-jun fusion protein following C_2-ceramide treatment of prostate cancer cells. Exogenous C_2-ceramide was found to be a strong activator of JNK1 activity in prostate carcinoma cells as determined by phosphorylation of c-jun substrate (Fig. 7). Ectopic expression of bcl-2 protein in the LNCaP-bcl-2 cells effectively impaired the catalytic activation of JNK1 by exogenous C_2-ceramide (50 μM) at 30 min and high dose C_2-ceramide (100 μM) at 20 and 30 min, in comparison to LNCaP-C vector control cells (Fig. 7A).

A phospho-specific antibody that recognizes phospho-JNK1 was also used to assess the extent of JNK1 activation in cells stimulated C_2-ceramide. Cell lysates prepared from LNCaP-C cells or LNCaP-bcl-2 cells stimu-

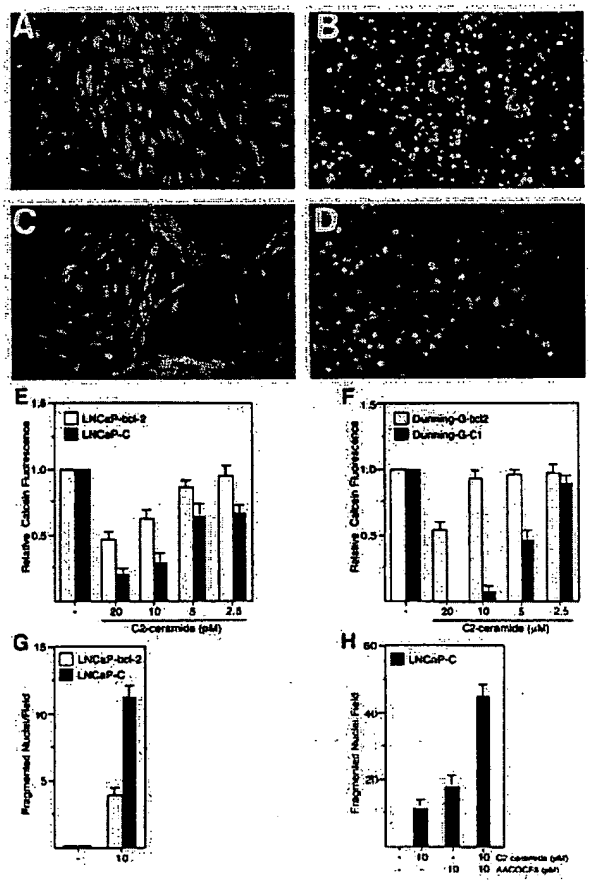

FIG. 6. Induction of apoptosis in prostatic carcinoma cells by cell permeable C_2-ceramide: Bcl-2-mediated protection against C_2-ceramide-mediated apoptosis. Cells were loaded with CAM and treated with 20 μM C_2-ceramide or ethanol in medium containing 0.5% serum and examined by epifluorescence microscopy at 24 h. Dunning-G-C1 cells treated with ethanol (A) and C_2-ceramide (B). Dunning-G-bcl-2 cells treated with ethanol (C) and C_2-ceramide (D). Cell viability was determined after 24 h by loading the cells with CAM and assessing calcein fluorescence intensity for LNCaP-C and LNCaP-bcl2 cells (E) and Dunning-G-C1 and Dunning-G-bcl-2 (F). Cells displaying the nuclear alterations of apoptosis were quantitated following uptake of ETHD in LNCaP-C and LNCaP-bcl-2 cells treated with 10 μM C_2-ceramide at 24 h (G). Apoptotic ETHD-loaded nuclei were also quantitated in LNCaP-C cells treated with 5 μM C_2-ceramide and/or 5 μM $AACOCF_3$ at 24 h (H). Columns, mean; bars, standard deviation.

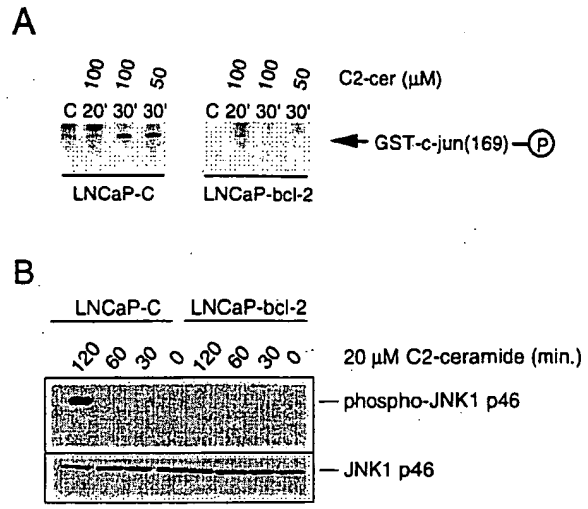

FIG. 7. Inhibition of ceramide-induced JNK1 activation by bcl-2. LNCaP-C and LNCaP-bcl-2 cells were treated with C_2-ceramide (50 or 100 μM) for the indicated times. Cells were lysed in JNK immunoprecipitation buffer, clarified of insoluble material, and immunoprecipitated using an anti-JNK1 antibody conjugated to Sepharose beads as described under Materials and Methods. The immune complex was washed four times using immunoprecipitation buffer and assayed as described. Phosphorylated c-jun(1-169)-GST was resolved in a 12.5% gel and detected by autoradiography. (B) Cell extracts were obtained from LNCaP-C or LNCaP-bcl-2 cells stimulated with C_2-ceramide (20 μM) for 0, 30, 60, and 120 min. Extracts were immunoblotted based on cell equivalency and probed with a mouse monoclonal anti-phospho-JNK antibody (G-7) followed by a secondary anti-mouse peroxidase-conjugated antibody for detection. These cell extracts were also run on another gel using equivalent loading volumes, immunoblotted, and probed with an anti-JNK antibody (C-17) to examine potential loading differences. The major protein species recognized by the G-7 antibody migrated at 46 kDa, corresponding to the size of JNK1. No discernible JNK2 phosphorylation was detected in these cells following C_2-ceramide stimulation during the chosen time period.

lated with C_2-ceramide (20 μM) for up to 2 h. Cell lysates were immunoblotted and probed with a monoclonal anti-phospho-JNK antibody that recognizes JNK1 or JNK2 specifically phosphorylated at residues Thr-183 and Tyr-185. In agreement with the ICKA, ectopic expression of bcl-2 in LNCaP prostatic carcinoma cells imposes a strong block to p46 JNK1 activation by C_2-ceramide (Fig. 7B). Discernible phosphorylation of p54 JNK2 was not observed with this particular stress in these cells.

Regulatory roles of PKC in C_2-ceramide-induced apoptosis in prostate carcinoma cells. Based on experiments involving protein kinase C (PKC) activators (TPA, 12-*O*-tetradecanoylphorbol-13-acetate) and inhibitors (calphostin C), PKC has been implicated in the regulation of ceramide-mediated apoptosis [39] and sphingosine-1-phosphate-mediated cell survival [40]. To inhibit PKC activity we used calphostin C, a relatively specific plasma membrane permeable inhibitor of PKC (IC_{50} PKC = 50 nM; IC_{50} PKA, cGMP-dependent kinase, src kinase > 25 μM). For these experiments cells were prestimulated with calphostin C for 30 min followed by costimulation with C_2-ceramide/calphostin C. Calphostin C alone signaled apoptosis in LNCaP prostate carcinoma cells through a bcl-2 inhibitable pathway. Control LNCaP cells displayed an approximately 2.7-fold greater sensitivity to apoptosis using combined calphostin C/C_2-ceramide stimulation (Fig. 8; $P \leq 0.01$). LNCaP-bcl-2, compared to LNCaP-C cells, were significantly more resistant to cell death induction by calphostin C/C_2-ceramide ($P \leq 0.01$). Cells stimulated with C_2-ceramide (10 μM) in the presence of TPA (25 nM), a potent activator of PKC, showed a significant reduction in C_2-ceramide induced apoptosis (Fig. 8). TPA (25 nM) in the absence of C_2-ceramide did not significantly signal apoptosis, although TPA at higher concentrations (≥100 nM) did have a significant cytotoxic effect on the LNCaP prostate carcinoma cells using the same experimental conditions (data not shown).

Regulatory roles of arachidonate in C_2-ceramide-induced apoptosis in prostate carcinoma cells. Costimulation of C_2-ceramide (10 μM)-treated cells with AA (10 μM) had a significant protective effect ($P \leq 0.01$) on C_2-ceramide-induced apoptosis. From these data we conclude that the AA pathway(s) for cell survival likely serves as a counterbalance lipid signaling system to the ceramide pathway for apoptosis. Further experiments

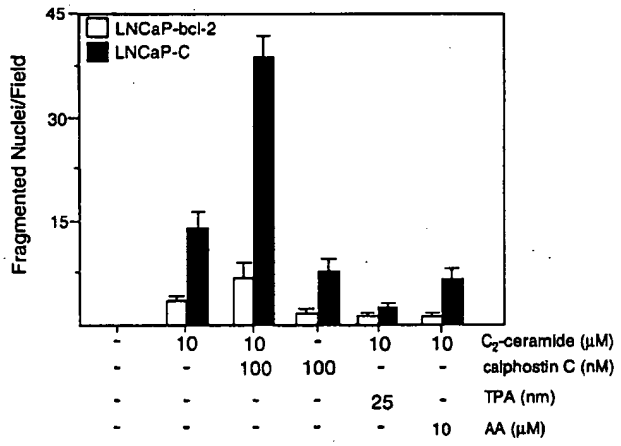

FIG. 8. Influences of TPA, calphostin C, and AA on C_2-ceramide-induced apoptosis. LNCaP-C and LNCaP-bcl-2 cells were prestimulated with TPA, calphostin C, or AA at the indicated concentrations for 30 min prior to costimulation with the following reagent combinations; TPA/C_2-ceramide, calphostin C/C_2-ceramide, or AA/C_2-ceramide. After 24 h cells were loaded with ETHD as described. Apoptosis was determined by counting cells with a pyknotic nuclear ETHD staining pattern in 4 randomly chosen fields per well. The experiment was performed in triplicate on two separate occassions. Columns, mean; bars, standard deviation.

will be designed to examine whether other AA-derived metabolites can oppose apoptosis signaling by ceramide.

DISCUSSION

Bcl-2 is characterized by its ability to insulate cells against diverse exogenous and endogenous stresses. Because AA metabolism generates different bioactive eicosanoids, it may represent a central switch point for signal transduction by lipid second messengers. AA also induces a number of activities independent of eicosanoids, suggesting AA may mediate a direct second messenger function [16]. Depletion of AA by inhibition of $cPLA_2$ would be predicted to not only impair signal transduction by bFGF, EGF, IFN-α, and IFN-γ among others but to also affect cell viability. Thus, AA could represent an important mediator of cell survival signal transduction which could be associated with bcl-2 function. We investigated this premise in prostatic carcinoma cells using $AACOCF_3$, a structural analog of AA, which is a selective inhibitor of $cPLA_2$ [28–30]. Application of $AACOCF_3$ to LNCaP or Dunning-G cells resulted in apoptosis induction. Expression of bcl-2 protein in either cell line provided significant protection against apoptosis induction by $AACOCF_3$. Furthermore, coincubation with free arachidonate effectively nullified the induction of apoptosis by incubation with $AACOCF_3$.

Since impairment of AA metabolism at the level of $cPLA_2$ would effectively block reincorporation of $cPLA_2$-released AA into plasma membrane phospholipid or the shunting of AA into the cyclooxygenase-, lipoxygenase-, or cytochrome P450-pathways it is essential to examine whether inhibition of these enzymes effects cell viability also. We examined whether the lipoxygenase or cyclooxygenase pathways might be involved using ETI and indomethacin. ETI is a selective inhibitor of lipoxygenase over cyclooxygenase in whole cells [31, 32]. At doses less than 10 μM for ETI, we observe no effect on the viability of the LNCaP or Dunning-G cells; however, incubation at 20 μM ETI resulted in a >99% reduction in cell viability in control cells. These findings suggest, but do not prove, that ETI-mediated apoptosis is a consequence of the inhibition of the 12- and 15-LOX enzymes. In Dunning-G or LNCaP cells transfected with bcl-2, cell viability was enhanced by 9.5- or 18-fold, respectively. Similarly, it has been recently reported that antisense LOX can induce apoptosis in rat Walker 256 carcinosarcoma cells [38]. The possibility remains, although it is perhaps unlikely, that the protective advantage conferred by bcl-2 is not directly related to AA but may be related to cellular alterations associated with AA metabolism.

It is considered that PGs are exclusively derived from AA via the cycloxygenase pathway I (COX I) or the cyclooxygenase pathway II (COX II). The 5-, 12-, and 15-LOX pathways are exclusively responsible for the biosynthesis of the HETEs, diHETEs, HPETEs, HEPA, KETE, various leukotrienes, and other eicosanoids. The cytochrome P-450 pathway is involved in the generation of HETEs, EETs, DHET, and other eicosanoids, but not PGs. Also, to the best of our knowledge, no reports have described the interconversion of eicosanoids derived from the LOX I or the LOX II pathways into PGs, suggesting that these pathways serve discrete cellular functions. Thus, we strongly believe that the LOX pathways, and in particular the 12- and 15-LOX pathways, strongly signal for cell survival. The role of prostaglandins is likely to involve other aspects of cell behavior.

The ability of bcl-2 to inhibit ceramide-mediated apoptosis in prostate carcinoma cells is an important observation and is consistent with observations showing that bcl-2 can inhibit ceramide-mediated cell death in leukemia cells [41]. Since, the MEKK/JNKK/JNK pathway is activated by multiple unrelated cell stress signals [15, 25], it seems reasonable to speculate that bcl-2 imposes a block to the activation of one or more of the enzymes involved in this pathway. In fact, we demonstrate that enforced expression of bcl-2 in prostate carcinoma cells blocks the activation of the stress-activated protein kinase JNK1 by ceramide. This finding is also consistent with the recent observation that bcl-2 both inhibits JNK activation and apoptosis following nerve growth factor (NGF) deprivation in PC12 pheochromocytoma cells [42]. Therefore, the ability of bcl-2 to regulate JNK activation may be a general feature of bcl-2 activity and provides a possible mechanistic basis for bcl-2 protection from cell stresses known to activate the sphingomyelin cycle.

Recently, it has been shown that the growth factor-linked ERK (MAPK) kinase pathway and cytokine/stress-responsive JNK-p38 kinase pathways may represent opposing pathways for cell viability and apoptosis, respectively, in PC12 pheochromocytoma cells following nerve growth factor (NGF) deprivation [43]. Importantly, it is known that bcl-2 can protect sympathetic neurons against apoptosis following NGF deprivation [42, 44]. Similarly, AA metabolism is essential for mitogenic signaling via the epidermal growth factor receptor (EGFR) in 3T3 fibroblasts [45]. Thus, diminished release of AA due to inhibition of $cPLA_2$ might be expected to result in the inhibition of mitogenic/survival signals initiated by ligand-bound receptors.

Although a matter of interest, the manner in which the AA and ceramide signaling pathways may be coordinately regulated remains speculative. In this regard, however, protein kinase C (PKC) has been directly implicated in opposing the ceramide pathway for apoptosis in that ceramide-mediated apoptosis is inhibited by

simultaneous application of PKC activators, including diacylglycerol (DAG) and TPA [24]. In so much as AA and other free fatty acids are involved in activating PKC [40], it is reasonable to speculate that the actions of ceramide and arachidonate could be coordinated at the level of PKC activity. Recent experimental evidence in support of this is provided by the demonstration that chelerythrine chloride and calphostin C, two selective inhibitors of PKC, could result in ceramide-mediated apoptosis [41]. Furthermore, phorbol esters which impede apoptosis signaling by ceramide also simultaneously activate $cPLA_2$, considered the rate limiting enzyme in AA mobilization [17]. Such a system would enable a cell to quickly respond to the balance of cell survival and apoptosis signals modulated by growth factor receptor/ligand interactions. One mechanism by which a PKC activator impedes C_2-ceramide-induced apoptosis may reflect the capacity of PKC to activate sphingosine kinase, the rate-limiting step in sphingosine-1-phosphate (S1P) biosynthesis. A recent report suggests that S1P directly opposses the ceramide pathway for apoptosis perhaps by stimulating the MAPK survival signaling pathway (40).

Our findings denote that the ceramide and AA/HETE pathways represent opposing bioactive lipid second messenger systems involved in the regulation of prostate carcinoma cell death which can be modulated by bcl-2. The net balance between these pathways would appear to represent an important determinant for cell survival or death. These findings further suggest that pharmacologic manipulation of the pathways for bioactive lipid metabolism might be used to clinical advantage.

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Phospholipase A_2 Inhibitors as Potential Anti-Inflammatory Agents

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Abstract: Phospholipase A_2 (PLA_2)-catalyzed hydrolysis of membrane phospholipids results in the stoichiometric production of a free fatty acid, most importantly arachidonic acid, and a lysophospholipid. Both of these phospholipid metabolites serve as precursors for inflammatory mediators such as eicosanoids or platelet-activating factor (PAF). Since it was initially discovered that non-steroidal anti-inflammatory drugs inhibit prostaglandin synthesis, a vast amount of drug development has been performed to selectively inhibit the production of the inflammatory metabolites of arachidonic acid while preserving their protective role. This research has culminated in the development of selective cyclooxygenase-2 (COX-2) inhibitors that act on the inducible, inflammatory COX enzyme, but do not affect the constitutive prostaglandin synthesis in cells that is mediated via COX-1.

The development of PLA_2 inhibitors as potential anti-inflammatory agents has also been extensively pursued since the release of arachidonic acid from membrane phospholipids by PLA_2 is one of the rate-limiting factors for eicosanoid production. In addition to the production of eicosanoids, PLA_2-catalyzed membrane phospholipid hydrolysis is also the initiating step in the generation of PAF, a potent inflammatory agent. Thus, inhibition of PLA_2 activity should, in theory, be a more effective anti-inflammatory approach. However, developing an inhibitor that would be selective for the production of inflammatory metabolites and not inhibit the beneficial properties of PLA_2 has so far proved to be elusive. This review will focus on agents used currently to inhibit PLA_2 activity and will explore their possible therapeutic use.

CLASSIFICATION OF PHOSPHOLIPASE A_2 ENZYMES

PLA_2 comprise an expanding family of distinct enzymes that can be classified into three main types, secretory ($sPLA_2$), cytosolic, Ca^{2+}-activated ($cPLA_2$) and Ca^{2+}-independent ($iPLA_2$) (Table 1, [1-5]). The PLA_2 enzymes within each type have been further classified into groups and sub-groups, based on their amino acid sequences (Table 1, [5]). At this time, 11 groups (I-XI) have been identified, with each group having at least one member and a majority containing two or more members. The three types of PLA_2 have been shown to coexist in mammalian cells and may interact with each other.

Secretory PLA_2 isoforms have low molecular weight (13-18 kDa), require the presence of millimolar concentrations of Ca^{2+} for hydrolysis of phospholipids, do not demonstrate a preference for specific *sn*-2 fatty acids, but selectively hydrolyze ethanolamine phospholipids. All $sPLA_2$ characterized to date contain an α-helical amino-terminal segment, a glycine-rich Ca^{2+}-binding loop and an active site His^{48} residue (Table 2, [6-10]). Since $sPLA_2$ requires millimolar Ca^{2+} concentrations, it was thought to act extracellularly and hydrolyze phospholipids that were present on the outside bilayer of the membrane. However, recent studies have indicated that some $sPLA_2$ isoforms may be internalized by caveolae [11] or may act within intracellular organelles in which there is a local concentration of Ca^{2+} high enough for the $sPLA_2$ catalytic activity [12]. These recent studies thus provide evidence for an intracellular role for $sPLA_2$.

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Cytosolic PLA_2 is expressed constitutively in most cell types. It is an 85 kDa protein that demonstrates selectivity for arachidonylated choline phospholipids [13-15] but does not demonstrate a difference in activity for substrates containing *sn*-1 acyl, vinyl ether and alkyl ether linkages when mammalian membranes are used as substrate [15]. Unlike $sPLA_2$, $cPLA_2$ does not require the presence of Ca^{2+} at the active site for catalytic activity (Table 2). However, in response to an increase in intracellular Ca^{2+}, $cPLA_2$ is translocated from the cytosol to intracellular membranes, primarily the nucleus and endoplasmic reticulum [16-18] and associates with the membrane phospholipids at these sites through a Ca^{2+}-dependent lipid binding domain [19]. The activity of $cPLA_2$ is increased by phosphorylation of Ser^{505} by protein kinase C and MAP kinases [20-26]. The most recently described member of the $cPLA_2$ family was found to be a smaller size (61 kDa) than the previously described $cPLA_2$ enzymes and has no catalytic requirement for Ca^{2+}[27]. However, since its sequence homology is closer to the $cPLA_2$ enzymes rather than the $iPLA_2$ enzymes, it has been categorized as a $cPLA_2$ whilst possessing catalytic features similar to $iPLA_2$ [27].

There are several forms of intracellular $iPLA_2$ widely distributed in cells and tissues with unique subcellular localization and function [28-40]. These isoforms differ from $sPLA_2$ and $cPLA_2$ isoforms in having no Ca^{2+} requirement for catalysis or phospholipid substrate binding (Table 2) and possess differential sensitivity to inhibitors (Table 1). $iPLA_2$ in mammalian cells has been shown to be involved in both arachidonic acid release and phospholipid remodeling which appears to be dependent upon the cell type and stimulus [24, 41, 42]. A subgroup of $iPLA_2$ enzymes are the PAF-acetylhydrolases (PAF-AH) that selective hydrolyze

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Table 1. Phospholipase A_2 Class and Group Nomenclatures, Molecular Weight Range and Commonly used Inhibitors for each Class

Class	Group	Alternative Nomenclature	Molecular Weight	Commonly Used Inhibitors*
Secretory	I A, B II A-F III V IX X XI A, B	$sPLA_2$	12-18 kD	LY311727 LY315920 PGBx PX-52 PX-18
Cytosolic	IVA IVB IVC	$cPLA_2\alpha$ $cPLA_2\beta$ $cPLA_2\gamma$	85 kD	AACOCF3, MAFP
Independent	VIA1 VIA2 VIB VIIA VIIB VIIIA VIIIB	$iPLA_2\alpha$ $iPLA_2\beta$ $iPLA_2\gamma$ PAF-AH PAF-AH II PAF-AH I α_1 PAF-AH I α_2	80-100 kD 45 kD 40 kD 26 kD	 BEL AACOCF3 MAFP

* Some inhibitors do not affect all groups within the class.

Table 2. Phospholipase A_2 Catalytic Site Characteristics

Class	Active Site Residue	Ca^{2+} Requirement
Secretory	Histidine	mM, required for hydrolysis
Cytosolic	Serine	μM, required for translocation of enzyme to an intracellular membrane
Independent	Serine	none

phospholipids with a short chain or oxidized acyl chain at the *sn*-2 position [43, 44].

CATALYTIC FEATURES OF PLA_2

In $sPLA_2$-catalyzed phospholipid hydrolysis, the catalytic calcium ion, bound to the conserved calcium binding loop, interacts simultaneously with water molecules and specific amino acid residues present at the enzyme active site. The role of the calcium ion at the active site is to properly orient the polar headgroup of the phospholipid substrate molecule and promote the redistribution of the π-orbital electron density at the *sn*-2 carbonyl leading to polarization of the carbonyl carbon-oxygen double bond. The polarization of the carbonyl group creates an electron deficient carbonyl carbon atom at the scissile *sn*-2 ester linkage. This promotes the nucleophilic attack by the oxygen atom of an adjacent water molecule at the electron deficient carbonyl carbon atom, leading to formation of a tetrahedral transition state intermediate. From the tetrahedral transition state, the lysophospholipid and fatty acid products are simultaneously generated and released following the concerted cleavage of the carbonyl carbon-*sn*-2 oxyester bond and intramolecular proton transfer facilitated by a "proton relay system" comprised of a histidine residue at position 48 and an aspartic acid residue at position 99. In order to maintain the proper spatial relationships for all the interacting elements at the active site, it is necessary to place tremendous constraints on the conformational flexibility of the $sPLA_2$ protein molecule. This is achieved by the formation of disulfide bonds which would aid in stabilizing protein tertiary structure and may represent the principal reason $sPLA_2$ possesses such a large number of disulfide bonds that are required for enzyme activity [45, 46]. The properties of interfacial recognition and catalysis (ie high affinity binding to and preferential hydrolysis of aggregated phospholipid substrates) are probably, in part, a manifestation of the fact that only at the substrate interface is the effective concentration of calcium, water and phospholipid substrate sufficiently high and the random molecular motion of these interacting elements sufficiently restricted to permit the rapid assembly of the catalytic complex needed for expression of maximal enzyme activity by $sPLA_2$.

The intracellular $cPLA_2$ and $iPLA_2$ enzymes operate via the formation of an acyl-enzyme intermediate [19, 40, 47,

48]. They do not require calcium ions for catalysis [19, 40, 47, 48] and function in a reducing environment inside the cell where disulfide bond formation is not favored [49]. The proposed mechanism for phospholipid hydrolysis by intracellular PLA_2 involves two sequential nucleophilic displacement reactions. The first represents a committing step that leads to formation of an acyl-enzyme intermediate and the second nucleophilic displacement reaction involves hydrolysis of the acyl-enzyme thioester (or O-acyl ester) group. The two successive nucleophilic displacement reactions are accompanied by the sequential release of the lysophospholipid product followed by the fatty acid product. Based on the ability of intracellular PLA_2 to exhibit activity with a wide variety of different substrates, the conformation of the enzyme active site is most likely sufficiently flexible to permit the utilization of different phospholipid and subclasses as alternative substrates.

Because of the increased conformational flexibility of intracellular PLA_2 which allows the enzyme to accomodate a wide variety of different substrates and to manifest other enzyme activities (eg lysophospholipase activity) in addition to PLA_2 activity, it would be expected that these enzymes would exhibit few absolute requirements with regard to amino acid sequence and could potentially exhibit a tremendous degree of amino acid sequence diversity. This is supported by the lack of significant sequence homology between $cPLA_2$ [38, 50-52] and $iPLA_2$ [53-57]. Sequence diversity among intracellular PLA_2 isoforms would allow for the synthesis of enzymes with different catalytic features. Accordingly, the cell-specific expression of different intracellular PLA_2 genes would permit the synthesis of proteins with regulatory features and substrate preferences specifically tailored to the phospholipid composition and physiologic function of the cell.

MEASUREMENT OF PLA_2 ACTIVITY

Since all types of PLA_2 catalyze the same enzymatic reaction, it can be extremely difficult to determine the activity of each in cells with multiple PLA_2 isoforms. The activity measurements made in any assay system depend upon multiple variables including the presence of calcium, the incubation time and temperature used, the variation in the structure of the phospholipid substrate (e.g., the *sn*-2 fatty acid, fatty acid linkage at the *sn*-1 position, polar headgroup at the *sn*-3 position), substrate concentration and the presence or absence of co-factors.

Attempts have been made to develop assay systems in which the activity of each type of PLA_2 can be determined individually with minimal or no contribution of activity from the other types [58, 59]. Measurement of activity in the absence of Ca^{2+} should be the most specific assay for $iPLA_2$ since both $cPLA_2$ and $sPLA_2$ require Ca^{2+} for phospholipid substrate hydrolysis and consequently only $iPLA_2$ activity should be measured in the presence of EGTA. For example, based on its Ca^{2+}-independent enzymatic activity and its stabilization by ATP, purified $iPLA_2$ has been measured in the absence of Ca^{2+} and in the presence of ATP using (16:0, [^{14}C]16:0) phosphatidylcholine as the phospholipid substrate. These assay conditions result in 10, 000-fold higher values for $iPLA_2$ activity when compared to the activity of $cPLA_2$ or $sPLA_2$ [58]. $cPLA_2$ activity can be measured selectively under assay conditions that are optimized to exploit its substrate selectivity for arachidonylated phospholipids and its activation by PIP_2 [58]. $sPLA_2$ activity can be measured selectively based on its dependence on anionic lipids for membrane association and its substrate preference for ethanolamine phospholipids [58]. These assay conditions were optimized using purified PLA_2 isoforms. However, in biological samples there will be multiple PLA_2 enzymes that may contribute to activity measurements and cellular inhibitors and activators of PLA_2 may also be present.

Ideally, PLA_2 activity should be measured using multiple endogenous substrates (e.g., plasmenylcholine, alkyl acyl glycerophosphorylcholine and phosphatidylcholine) in the absence and presence of calcium and under assay conditions that can exploit individual properties of each isoform. In addition, preliminary studies would have to be carried out when measuring PLA_2 activity in a novel cell system or tissue to ensure that PLA_2 activity is being measured under conditions which show linear reaction velocities with respect to time and enzyme concentration for each phospholipid substrate used. To illustrate the need for the use of multiple substrates and stringent assay conditions, we employed several previously described assays to determine PLA_2 activity in cytosolic and membrane protein fractions isolated from human endothelial cells [59]. We measured endothelial cell PLA_2 activity in the presence and absence of calcium using plasmenylcholine, phosphatidylcholine and alkyl acyl glycerophosphorylcholine substrates. We found a large discrepancy in PLA_2 activity measured using different assay systems, which was dependent upon the concentration and type of phospholipid substrate used, and the incubation time [59]. Most importantly, activation of endothelial cell PLA_2 by thrombin was detectable only when plasmenylcholine was used as the phospholipid substrate, with no increased PLA_2 activity measured with phosphatidylcholine or alkyl acyl glycerophosphorylcholine substrates [59].

INHIBITION OF PLA_2 ACTIVITY

Many PLA_2 inhibitors originally designed to be selective for a specific PLA_2 isoform have been found subsequently to inhibit more than one isoform as new information about specific PLA_2 isoforms is discovered. Additionally, and more importantly, some of these inhibitors have been shown to possess actions distinct from PLA_2 inhibition (see below). Historically, the compounds that were originally used to inhibit PLA_2 activity included antimalarial drugs, aminoglycosides and polyamines. Subsequently, it was discovered that these agents do not act on the enzyme, but disrupt the interaction between the enzyme and either its phospholipid substrate or calcium ions [60]. These "classic" PLA_2 inhibitors are rarely employed today and thus this review will focus on the development of the newer, more specific inhibitors of PLA_2 isoforms.

The best characterized specific $sPLA_2$ inhibitor to date is 3-(3-acetamide-1-benzyl-2-ethylindolyly-5-oxy) propane sulfonic acid (LY311727) [61]. At low nanomolar concentrations, this inhibitor binds and inhibits both Group IIA $sPLA_2$ (found in some snake venom, human platelets and human synovial fluid) and Group V $sPLA_2$ (found in P388D$_1$

macrophages), but does not inhibit Group 1 $sPLA_2$ (found in porcine and human pancreas). LY311727 resides in the hydrophobic channel of $sPLA_2$, resulting in structural changes in the channel to accommodate the inhibitor, and interacts directly with the active site [61]. A more recently described analog of LY311727, ([[3-(amino-oxoacetyl)-2 - ethyl - 1 - (phenylmethyl) - 1*H* - indol - 4 - yl] oxy] acetate (LY315920) has been shown to be an extremely potent (IC_{50}=9nM) and selective inhibitor of Group IIA $sPLA_2$ that also acts at the active site [62].

Another family of compounds that are selective for $sPLA_2$ have at least two fatty moieties and contain at least one unsaturated double bond. The fatty moieties may be different from each other in several features including, but not limited to, chemical composition, functional groups, the degree of unsaturation, and the length of the hydrocarbon chain. The compounds may also have at least one organic group, one active acid group or any salt form or ionized form thereof. The first described inhibitor in this group was PGBx, a prostaglandin oligomer , that was found to inhibit $sPLA_2$ activity and to block release of arachidonic acid from human neutrophils in 1989 [63, 64]. Subsequently, a related compound, PX-52, a fatty acid polymer, was found to inhibit a variety of $sPLA_2$ isoforms at IC concentrations between 1.0 and 3.7 μM [65]. However, this compound also inhibited $cPLA_2$ from U937 cells with an IC_{50} of 5 μM [65]. The newest member of this family, PX-18 (2-[N, N-bis(2-oleoyloxyethyl)amine]-1-ethanesulfonic acid) inhibits human disc $sPLA_2$ with an IC_{50} of less than 1 μM but demonstrates no measurable inhibition of recombinant $cPLA_2$ (personal communication, Richard Berney Associates, LLC)

Two mechanism-based inhibitors, arachidonyl trifluoromethyl ketone ($AACOCF_3$) and methyl arachidonyl fluorophosphonate (MAFP) were originally developed as specific inhibitors for $cPLA_2$, but were subsequently found to inhibit $iPLA_2$ at similar concentrations [66-68]. The two compounds have an arachidonyl tail coupled to a serine reactive group and they act by competing with endogenous phospholipid molecules for the active catalytic site on the PLA_2 enzyme. $AACOCF_3$ is a tight-binding reversible inhibitor that exhibits slow binding with $cPLA_2$, but not with $iPLA_2$ [66]. It inhibits both $cPLA_2$ and $iPLA_2$ by forming a stable hemiketal with the active site serine residues [66, 67]. MAFP has been shown to irreversibly inhibit both $cPLA_2$ and $iPLA_2$, possibly by phosphorylation of the active site serine residue [68]. Neither MAFP nor $AACOCF_3$ inhibit $sPLA_2$ isoforms. Since both AACOCF3 and MAFP inhibit both $cPLA_2$ and $iPLA_2$, several strategies have been employed using combinations of inhibitors to try to distinguish between $cPLA_2$ and $iPLA_2$ inhibition by either of these agents. For example, since bromoenol lactone inhibits $iPLA_2$ selectively (see below), the difference in total PLA_2 activity measured in the presence of BEL with MAFP or $AACOCF_3$ compared to that measured in the presence of BEL alone would represent $cPLA_2$.

Subsequently, a series of fatty alkyl trifluoromethyl ketones and methyl fluorophosphonates has been synthesized and tested for specificity for $iPLA_2$ or $cPLA_2$ isoforms with a view to developing more specific inhibitors [69]. This study demonstrated that by synthesizing analogs of MAFP or $AACOCF_3$ it is possible to develop inhibitors that are more selective for either $iPLA_2$ or $cPLA_2$. These analogs may be of considerable value in future studies. The most selective $cPLA_2$ inhibitor described to date is a pyrrolidine-based inhibitor, pyrrolidine-1. This inhibitor has been reported to inhibit Group IVA $cPLA_2$ by a specific mechanism involving a protein-inhibitor interaction [70]. The IC_{50} for Group IV A $cPLA_2$ inhibition is 0.07 μM when (16:0, 20:4) phosphatidylcholine vesicles are used as substrate, but is 1.2 μM for Group IV C $cPLA_2$ (a calcium-independent PLA_2) under similar assay conditions and is 8 μM for Group VI A-1 $iPLA_2$ in an assay using phosphatidylcholine substrate [70]. Because of its enhanced selectivity for $cPLA_2$, pyrrolidine-1 has been used in several recent studies to examine the role of $cPLA_2$ activity (71-73).

The most isoform-selective PLA_2 inhibitor to date is the $iPLA_2$ inhibitor, bromoenol lactone (BEL), which has been demonstrated to have 100-fold selectivity for $iPLA_2$ vs $cPLA_2$ and $sPLA_2$ isoforms [74]. Recently, Gross and coworkers have resolved racemic BEL into its individual enantiomeric constituents and shown that these enantiomers can distinguish between individual isoforms of $iPLA_2$ [75]. Group VI A-1 $iPLA_2$ is selectively inhibited by (S)-BEL, whereas (R)-BEL selectively inhibits Group VI B $iPLA_2$. This study highlights the usefulness of chiral mechanism-based inhibitors to further discriminate between PLA_2 isoforms [75]. However, although BEL selectively inhibits $iPLA_2$ activity at low micromolar concentrations (0.5-3 μM), it has been shown to inhibit phosphatidate phosphohydrolase, an enzyme that converts phosphatidic acid into diacylglycerol, at higher concentrations (IC_{50}=8 μM) [76]. Thus appropriate concentrations need to be used in studies that do not measure PLA_2 activity directly.

Due to the problems associated with the specificity of pharmacologic inhibitors, several molecular biology techniques have been used to support data obtained using pharmacologic inhibitors. These techniques include inhibition of PLA_2 activity using antisense oligonucleotides [77-81], overexpression of a specific PLA_2 isoform [82-84], and the development of transgenic [85, 86] or knockout mice [87-94] to study the function of a specific isoform. In general, success with antisense inhibition of gene expression depends on balancing three factors: 1) efficient delivery, 2) potency and 3) specificity. Preliminary studies in human cell lines indicate that specific knock down of individual PLA_2 isoforms is possible. The utility of this approach for use with other isoforms is supported by the fact that the sequences encoding PLA_2 even within a single group can be vastly different ranging from only 8% protein sequence identity in human group IV to a maximum of 61% in group VIII. This relative lack of sequence identity assures that for any given PLA_2 hundreds of putative antisense sequences are available for comparison to minimize off-target effects and maximize on target reduction of gene expression. Furthermore, the investigation of antisense inhibition of PLA_2 could lay the groundwork for discovery of a nucleic acid-based antithrombotic drug. The most well described PLA_2 knockout model is the $cPLA_2\alpha$ knockout mouse developed by Bonventre and co-workers that has been used in several published studies to investigate the role of $cPLA_2\alpha$ [95]. However, the knockout mouse may not be suitable for each

PLA_2 isoform as a model for studying human disease. This is illustrated by a recent study performed by Mancuso *et al.* that expressed $iPLA_2\beta$ in transgenic mice in a cardiac myocyte-specific manner [96]. Malignant ventricular arrhythmias were produced in the $iPLA_2\beta$ transgenic hearts in response to ischemia whereas ventricular arrhythmias in ischemic mouse hearts is rare [96]. Since malignant ventricular arrhythmias are common in ischemic human heart and other animal models, the $iPLA_2\beta$ transgenic mouse is a good model to use to study this phenomenon and demonstrates that $iPLA_2\beta$ directly contributes to ischemia-induced arrhythmogenesis [96]. However, this study illustrates that the presence of increased $iPLA_2$ activity in the $iPLA_2\beta$ transgenic mouse demonstrates ischemia-induced cardiovascular changes that are similar to those seen in the human heart, suggesting that the normal mouse serves as a knockout or knockdown model of the human myocardium due to its lower expression of $iPLA_2\beta$ and its lack of ischemia-induced arrhythmogenesis. The first $iPLA_2\beta$ knockout mouse has been recently described (97). To date, homozygous $iPLA_2\beta$ gene disruption has been shown to impair male reproductive ability by causing production of spermatozoa with reduced motility, but no other information about this model is currently available.

ROLE OF PLATELET-ACTIVATING FACTOR IN INFLAMMATION

Platelet-activating factor (PAF) is a potent lipid autacoid rapidly synthesized and presented on the surface of endothelial cells in response to a variety of agonists and conditions. Endothelial cell PAF production significantly contributes to the recruitment of leukocytes and monocytes to inflamed tissue by promoting the initial adherence to the endothelium [98] and thus can play a major role in the progression of inflammatory diseases [99]. Inflammation is a central feature of atherosclerosis and thrombosis, both processes in which PAF is intimately involved. PAF has been implicated in the development of myocardial ischemia-reperfusion injury and plays a role in hypotension and the cardiac dysfunction occurring in cardiovascular stress situations such as cardiac anaphylaxis, and hemorrhagic, traumatic, and septic shock syndromes.

The synthesis of platelet-activating factor (PAF) occurs through two pathways, the remodeling pathway and the *de novo* pathway. Evidence indicates that the remodeling pathway for PAF synthesis is activated during inflammation and hypersensitivity responses, whereas the *de novo* synthetic pathway is thought to be the source of PAF required for physiologic functions [99]. In recent studies, we have demonstrated that thrombin stimulation of human endothelial cells results in a time dependent increase in membrane-associated $iPLA_2$ activity. This results in increased lysoplasmenylethanolamine production that appears to be a requirement for PAF synthesis in thrombin stimulated endothelial cells, demonstrating that the remodeling pathway of PAF significantly contributes to PAF synthesis in thrombin-stimulated endothelial cells (Fig. **1**, [59, 98]).

The concentration of PAF in plasma and tissues is tightly regulated by the balance of its synthesis and degradation [100]. Since PAF is a potent inflammatory phospholipid metabolite, it is normally maintained at low concentrations by being rapidly degraded by PAF acetylhydrolase (PAF-AH, Fig. **1**). PAF-AH is classified as a cytosolic calcium-independent PLA_2 that preferentially hydrolyzes phospholipids with short chain or oxidized fatty acids at the *sn*-2 position [43, 44, 100, 101]. It hydrolyzes the acetyl group at the *sn-2* position of PAF to form the biologically inactive lyso-PAF [100]. PAF-AH causes PAF to lose its ability to activate cell polarization and cell spreading in vitro and abolishes the inflammatory effects of PAF that involve adherence of leukocytes to the vasculature [102]. PAF-AH exists as both an intracellular molecule and as a secreted enzyme. The plasma form of PAF-AH, a scavenger of both PAF and oxidized phospholipids, is generally found in a complex with low- and high-density lipoproteins [99, 103]. PAF-AH is thought to play a role in atherosclerosis by degrading PAF-like oxidized phospholipids that bind PAF receptors [104] and are implicated in the pathogenesis of atherosclerosis. [105].

Interestingly, we have recently demonstrated that pretreatment of endothelial cells with methyl arachidonyl fluorophosphonate (MAFP, an inhibitor of cytosolic Ca-dependent and -independent PLA_2 isoforms) [68] increases basal and thrombin-stimulated PAF production as a direct result of the inhibition of PAF-AH activity (Fig. **1**, [98]). The increase in PAF production in MAFP-pretreated, thrombin-stimulated endothelial cells is accompanied by increased expression of P-selectin (CD62P) on the endothelial cell surface and by increased neutrophil adherence to the endothelial cell monolayer. We investigated the ability of BEL and PX-18 to inhibit PAF-AH to determine whether $iPLA_2$ or $sPLA_2$ inhibitors also inhibit PAF-AH. When human coronary artery endothelial cells (HCAEC) were incubated with increasing concentrations of MAFP, BEL or PX-18, we did not see any significant inhibition of PAF-AH with either BEL or PX-18, but MAFP caused a concentration-dependent inhibition of PAF-AH (Fig. **2**). These data suggest that MAFP could act as a pro-inflammatory agent, prolonging the inflammatory response and increasing the recruitment of inflammatory cells to areas of injury [98].

ROLE OF EICOSANOIDS IN INFLAMMATION

Activation of both PLA_2 to release arachidonic acid from membrane phospholipids, and COX, which converts arachidonic acid to the intermediate prostaglandin precursor PGH_2, represent the two crucial rate limiting steps for the prostaglandin biosynthetic pathway. Since there are both immediate and delayed prostaglandin biosynthetic responses, it is thought that different combinations of PLA_2/COX enzymes are involved. It is proposed that production of prostaglandins within minutes is mediated by COX-1, the constitutive isoform, and more delayed prostaglandin production occurs via COX-2, the inducible isoform [106-110]. However, whether distinct PLA_2 isoforms are utilized specifically with each COX isoform remains controversial.

$cPLA_2\alpha$ has been proposed to play a central role in immediate and delayed eicosanoid production, primarily since it has been shown that $cPLA_2\alpha$ knockout mice produce

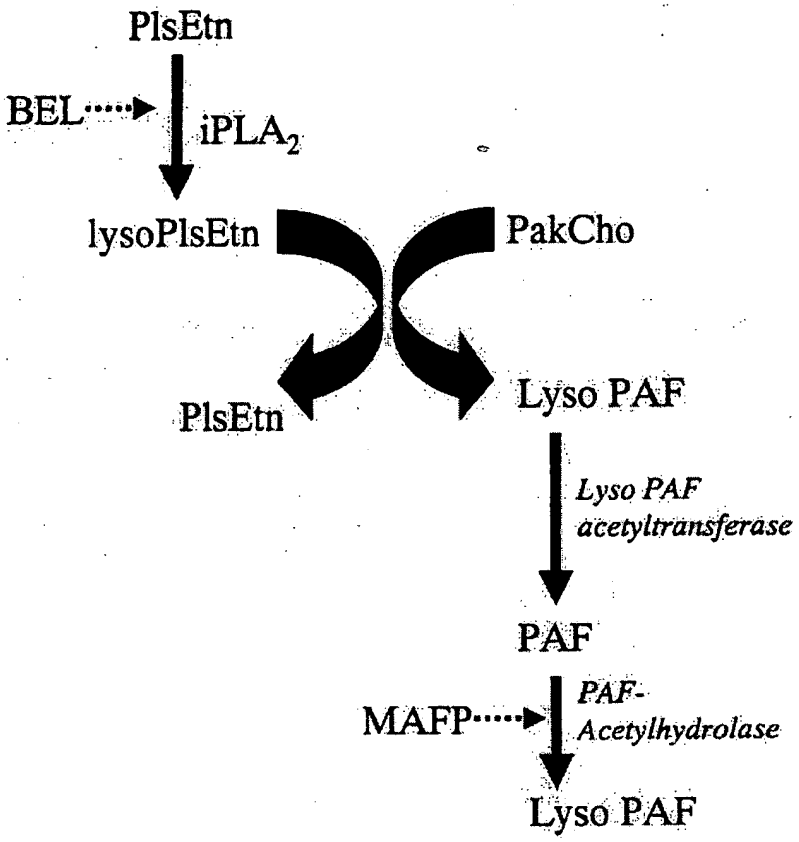

Fig. (1). The remodeling pathway for PAF production is initiated by phospholipase A_2 (PLA_2)-catalyzed hydrolysis of membrane phospholipids. In thrombin-stimulated endothelial cells, we have demonstrated that calcium-independent PLA_2 ($iPLA_2$) preferentially hydrolyzes plasmenylethanolamine (PlsEtn) to produce lysoplasmenylethanolamine (lysoPlsEtn) that acts as a lysophospholipid acceptor. The hydrolysis of the PlsEtn to lysoPlsEtn can be blocked by the $iPLA_2$ specific inhibitor bromoenol lactone (BEL). Transacylation between lysoPlsEtn and alkylacyl-glycerol-3-phosphocholine (PakCho) results in reacylation of lysoPlsEtn and the production of lyso-PAF. Lyso-PAF is then acetylated by acetyl-CoA:1-alkyl-*sn*-glycero-3-phosphorylcholine 2-*O*-acetyltransferase to produce PAF, which can be rapidly hydrolyzed by PAF-acetylhydrolase. Methyl arachidonyl fluorophosphonate (MAFP, an inhibitor of cytosolic Ca^{2+}-dependent and -independent PLA_2 isoforms) increases PAF production as a direct result of the inhibition of PAF-AH activity.

minimal eicosanoids in response to stimuli [111]. In response to an increase in intracellular Ca^{2+}, $cPLA_2\alpha$ translocates from the cytosol to the perinuclear membrane [17, 112], where COX and 5-lipoxygenase (5-LO) exist [113, 114]. Immediate production of prostaglandins may then involve a tightly coupled reaction between $cPLA_2\alpha$ and COX. The proposed catalytic mechanism for intracellular PLA_2 isoforms which allows formation of an acylenzyme intermediate (see above) would support the theory of a direct role for intracellular PLA_2 in immediate prostaglandin synthesis. For example, the presence of a long-lived acylenzyme intermediate would permit the inherent specificity of protein-protein interactions to target delivery of PLA_2 enzyme-bound arachidonate to other downstream elements in the pathway of eicosanoid production (47). The targeted movement of arachidonate present in a covalently bound form at the active site of a calcium-independent PLA_2 molecule would greatly increase the efficiency of arachidonic acid delivery. Although intracellular PLA_2 isoforms may be directly coupled to COX within the cell for eicosanoid production, several studies have suggested that immediate eicosanoid production involves both $cPLA_2$ and $sPLA_2$, with $cPLA_2$ being the activator of the response, but $sPLA_2$ providing the bulk of arachidonic acid release [113, 115, 116-118].

Evidence is accumulating that $cPLA_2\alpha$ is also involved in the delayed production of eicosanoids, however, it is unlikely that $cPLA_2\alpha$ is involved directly since the intracellular calcium concentrations would be low [116, 119, 120]. Instead, it has been proposed that activation of $cPLA_2\alpha$ is an $sPLA_2$ regulator and that $sPLA_2$ provides the arachidonic acid required for eicosanoid generation. After $cPLA_2$ is activated, the expression of both $sPLA_2$ and COX-2 are upregulated in delayed prostaglandin production [79, 81]. In an elegant study published recently [11], Balboa and co-workers have shown that group V $sPLA_2$ is present in

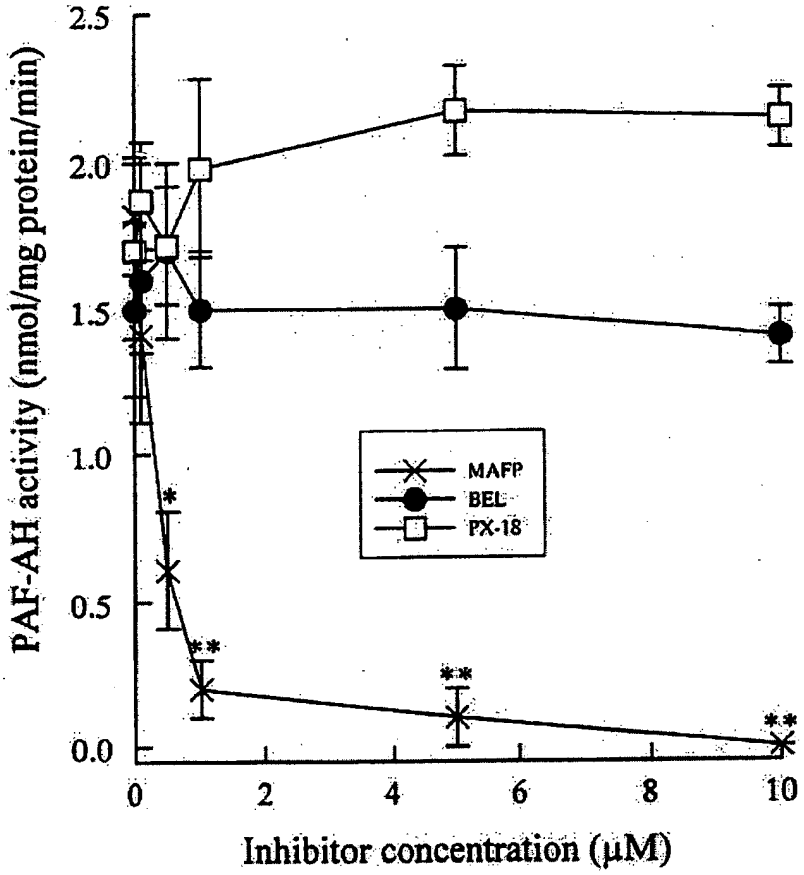

Fig. (2). PAF-acetylhydrolase (PAF-AH) activity measured in human coronary artery endothelial cells incubated with PLA_2 inhibitors for 10 mins. PAF-AH activity was significantly inhibited by methyl arachidonyl fluorophosphonate (MAFP) at concentrations as low as 0.5 μM, whereas bromoenol lactone (BEL) and PX-18 did not significantly change PAF-AH activity at concentrations as high as 10 μM. * $p<0.05$, ** $p<0.01$ when compared to controls. Values are mean ± SEM for 4 separate experiments. PAF-AH activity was measured as described previously in reference 98.

cytoplasmic granules in the perinuclear region of lipopolysaccharide (LPS)-treated $P388D_1$ macrophages. Since these granules contain caveolin-2, it was proposed in this study that they result from internalization of cell surface-associated $sPLA_2$ via a caveolae-mediated endocytosis. Furthermore, since caveolae are sites of calcium entry and storage, and the granules would protect $sPLA_2$ from the reducing environment in the cell, the encapsulation of $sPLA_2$ would also provide an ideal working environment for the enzyme, effectively rendering it active directly within the cell, where the COX enzymes can access free arachidonic acid. This scenario serves to provide an answer to the long-asked question of how extracellularly-acting $sPLA_2$ and intracellular COX enzymes could effectively be coupled to produce eicosanoids without the problem of excessive arachidonic acid trafficking between the outside and the inside of the cell. However, caveolin-2 is more of a Golgi marker than for caveolae [117, 118], thus the speculation that group V $sPLA_2$ is associated with caveolae may be premature.

Although the combination of $cPLA_2$/$sPLA_2$/COX has been shown to be responsible for immediate and delayed prostaglandin production in several studies, these studies have primarily been performed in monocyte and macrophage-like cell lines. Whether this holds true for all cell types under all conditions remains to be elucidated. In addition, although there is strong evidence to show that arachidonic acid release is dependent upon both $cPLA_2$ and $sPLA_2$ activity, there is no evidence regarding the directionality of this cross-talk between the two. In fact, since $cPLA_2$ displays remarkable selectivity for arachidonylated substrates whereas $sPLA_2$ does not, it could be expected that $cPLA_2$ activity is responsible for arachidonic acid release and that $sPLA_2$ activates $cPLA_2$, as has recently been suggested by Gelb and co-workers [12]. They have demonstrated that arachidonic acid release from human embryonic kidney (HEK) cells transfected with group II $sPLA_2$ is not due to its internalization and that the $sPLA_2$ resides in the Golgi in both HEK and CHO cells. They also provide evidence that arachidonic acid release by group II

$sPLA_2$ in transfected HEK and CHO cells occurs prior to secretion to the extracellular fluid, suggesting that Golgi-associated $sPLA_2$ can hydrolyze membrane phospholipids intracellularly and thus may not be dependent upon prior $cPLA_2$ activation [12]. There are studies that demonstrate that $cPLA_2$ activation may be a result of increased $sPLA_2$ [121, 122] or COX-2 [123] activity, thus the question of the directionality or sequence of $sPLA_2$/$cPLA_2$/COX activation is still not completely resolved.

Several published studies are now describing a possible role for $iPLA_2$ in the generation of eicosanoids. Tay and Melendez [124] have demonstrated that immunoglobulin G receptors (FcγR) are functionally coupled to $iPLA_2\beta$ for the release of arachidonic acid and the production of leukotriene B_4 and PGE_2. A role for $iPLA_2$ in eicosanoid generation was demonstrated in this study using BEL, $iPLA_2\beta$ antisense and depletion of intracellular Ca^{2+} with BAPTA and EDTA [124]. Murakami *et al.* [121] have transfected various PLA_2 and COX enzymes into HEK cells and examined functional coupling between them in immediate and delayed prostaglandin synthesis. They showed that arachidonic acid released by $cPLA_2\alpha$, group IIA $sPLA_2$ and group V $sPLA_2$ was converted to PGE_2 by both COX-1 and COX-2 during the immediate response and solely by COX-2 in the delayed response [123]. Ionophore-induced immediate PGE_2 generation was linked to $iPLA_2\beta$ and COX-1 activity, but no role for $iPLA_2$ was determined in the delayed response [123]. The authors suggest that $iPLA_2$ releases arachidonic acid in closer proximity to COX-1 than COX-2 or that $iPLA_2$-derived arachidonic acid was somehow inaccessible to COX-2. Finally, the authors demonstrated that extracellular $sPLA_2$ isoforms augmented PGE_2 production in neighboring cells, thus propagating the prostaglandin synthesis signal [123]. These studies illustrate that different PLA_2 isoforms within a cell type can play a role in prostaglandin synthesis in response to various stimuli and that the development of a selective PLA_2 inhibitor may be directed towards a cell type undergoing a specific response.

THERAPEUTIC POTENTIAL FOR PLA_2 INHIBITORS

Inhibition of specific PLA_2 isoforms is potentially an effective therapy for several inflammatory conditions and has been actively explored for several years. Despite this, there are no selective PLA_2 inhibitors clinically available to date. The identification and characterization of additional members of the expanding PLA_2 family and development of more selective PLA_2 inhibitors has not served to elucidate an anti-inflammatory role for PLA_2 inhibition as was previously hoped. In fact, the expanding field of PLA_2 research in some ways seems to complicate matters further with each passing year. For example, the development of a cell-impermeable $sPLA_2$ inhibitor that would protect the cell membrane from $sPLA_2$ activity without affecting vital intracellular PLA_2 activity was proposed to be an effective therapeutic avenue, but with the recently published data that suggest that $sPLA_2$ activity may occur within the cell, even this premise may prove to be too empirical [125].

As more information becomes available regarding the role of PLA_2 isoforms in different cells and tissues, it can be predicted that the ability to develop selective inhibitors will be enhanced. For example, as more information becomes available concerning the structure, functional sites and modulators of activity, selective PLA_2 inhibitors may be developed to specifically target functional groups on the enzyme other than the catalytic site or to target alternative intracellular sites. Additionally, studies that demonstrate inhibition of PLA_2 activity under experimental conditions may identify alternative avenues to explore for therapeutically suitable PLA_2 inhibitors. For example, in several studies, we have demonstrated that anthracyclines inhibit myocardial $iPLA_2$ activity at micromolar concentrations, thus it is reasonable to predict that development of anthracycline analogs that minimize cardiotoxicity whilst retaining PLA_2 inhibitory action could be useful anti-inflammatory agents.

One of the problems associated with the development of therapeutic PLA_2 inhibitors is separation of the physiological from the pathological properties of the enzymes. Clearly, PLA_2-catalyzed hydrolysis of membrane phospholipids plays a role in multiple beneficial and essential processes. The potential role of $iPLA_2$ inhibitors as inhalation or instillation therapy would seem to be ideal as maximal inhibition of PLA_2 activity could in theory be achieved whilst minimizing systemic side effects caused by inhibition of the beneficial function of PLA_2. PLA_2-catalyzed hydrolysis of membrane phospholipids provides the precursors for several cell signaling and biochemical responses under physiological conditions. In addition, PLA_2-catalyzed membrane phospholipid repair is essential following injury under several conditions. For example, PLA_2 plays an essential role in the detoxification of oxidized membrane phospholipids and thus systemic PLA_2 inhibition could augment cellular injury as a side effect of therapy.

An advantage of PLA_2 inhibitors over cyclooxygenase or lipoxygenase inhibitors should be the reduction of PAF production, however, our data obtained using MAFP suggests that inhibition of PAF acetylhydrolase is a major concern when developing an effective therapeutic agent (Figs. **1** & **2**). Clearly, if different PLA_2 enzymes are involved in eicosanoid generation dependent upon cell type and stimulus, it may be possible to develop PLA_2 inhibitors to selectively treat a disease process whilst preserving beneficial eicosanoid generation. However, there is still much left to be determined concerning the isoforms of PLA_2 that are involved in the generation of inflammatory mediators in different cell types in response to various stimuli. Additionally, and probably most importantly, some of the "selective" PLA_2 inhibitors available can also produce unexpected results suggesting that they are capable of being pro-inflammatory as well as anti-inflammatory and thus must be viewed with caution as potential anti-inflammatory agents.

In summary, one of the major challenges of using PLA_2 inhibitors as potential therapeutic agents is to optimize maximal efficacy with minimal side effects. The emergence of a growing body of literature more elegantly describing and characterizing PLA_2-catalyzed membrane phospholipid hydrolysis under experimental conditions can only serve to further our understanding of this complicated process. In the

future, the use of PLA_2 inhibitors may be governed more by the pharmaceutical aspect of therapy rather than the pharmacologic one. For example, targeting the inhibitors to a specific cell type by introducing a homing tag, using localized application rather than systemic or targeting the enzyme to recognize a specific intracellular biochemical event (such as a decrease in pH as would be seen in myocardial ischemia) may broaden the potential for the use of PLA_2 inhibitors. Finally, the emerging role of PLA_2 enzymes as membrane phospholipid repair enzymes under certain disease conditions poses the intriguing possibility that we may eventually be more interested in preserving PLA_2 activity than inhibiting it.

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